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Transcription controls growth, cell kinetics and cholesterol supply to sustain ACTH responses.

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Abstract

Chronic ACTH exposure is associated with adrenal hypertrophy and steroidogenesis. The underlying molecular processes in mice have been analysed by microarray, histological and immunohistochemical techniques. Synacthen infused for 2 weeks markedly increased adrenal mass and plasma corticosterone levels. Microarray analysis found greater than 2-fold changes in expression of 928 genes ($P < 0.001$; 397 up, 531 down). These clustered in pathways involved in signalling, sterol/lipid metabolism, cell proliferation/hypertrophy and apoptosis. Signalling genes included some implicated in adrenal adenomas but also upregulated genes associated with cyclic AMP and downregulated genes associated with aldosterone synthesis. Sterol metabolism genes were those promoting cholesterol supply (*Scarb1*, *Sqle*, *Apoa1*) and disposal (*Cyp27a1*, *Cyp7b1*). Oil red O staining showed lipid depletion consistent with reduced expression of genes involved in lipid synthesis. Genes involved in steroidogenesis (*Star*, *Cyp11a1*, *Cyp11b1*) were modestly affected ($P < 0.05$; < 1.3 -fold). Increased *Ki67*, *Ccna2*, *Ccnb2*, *Tk1* expression complemented immunohistochemical evidence of a 3-fold change in cell proliferation. Growth arrest genes, *Cdkn1a* and *Cdkn1c*, which are known to be active in hypertrophied cells, were increased > 4 -fold and cross-sectional area of fasciculata cells was 2-fold greater. In contrast, genes associated with apoptosis (eg *Casp12*, *Clu*,) were down-regulated and apoptotic cells (Tunel staining) were fewer ($P < 0.001$) and more widely distributed throughout the cortex.

In summary, long term steroidogenesis with ACTH excess is sustained by genes controlling cholesterol supply and adrenal mass. ACTH effects on adrenal morphology and genes controlling cell hypertrophy, proliferation and apoptosis suggest the involvement of different cell types and separate molecular pathways.

Introduction

Temporal control of adrenocortical responses to ACTH involves several processes, mediated by a common signalling system. *In vivo* and *in vitro* studies show that within five minutes, stress and ACTH cause increased adrenal corticosteroid release (Pearlmutter, et al. 1973; Walker, et al. 2015). Closer analysis of acute responses has demonstrated that activation of melanocortin receptors trigger cyclic AMP synthesis, leading to the synthesis of StaR which promotes the uptake of cholesterol into mitochondria (Artemenko, et al. 2001; Clark 2016; Simpson and Waterman 1983). Since steroid hormones are synthesised on demand, the availability of intramitochondrial cholesterol initiates steroidogenesis by providing substrate for the first rate-limiting enzyme in the steroidogenic pathway (Miller and Bose 2011). Long-term exposure to ACTH, requires changes in cholesterol supply steroidogenic enzyme expression and adrenocortical cell hypertrophy and hyperplasia which take place over hours, days and weeks to maintain steroid output at a continuous high level. Although many of these key processes involve non-genomic enzyme activation, transcriptional control is also important.

Long term supply of cholesterol substrate is maintained by *de novo* synthesis, by uptake from the circulation and by release from stored intracellular lipid droplets. Key steps in *de novo* synthesis are hydroxymethylglutaryl CoA reductase (*Hmgcr*), squalene epoxidase (*Sqle*) and various post-lanosterol reductase and dehydrogenase steps (Ness 2015). Plasma LDL and HDL cholesterol (Brown, et al. 1979; Gwynne and Strauss 1982) are also available for steroidogenesis via LDL (*Ldlr*) and scavenger receptor (*Scarb1*) respectively. Within the adrenal cortex, cholesterol is stored as an ester in lipid droplets or utilized for steroidogenesis depending on the balance between lipase and esterifying enzyme activity (Beckett and Boyd 1977; Kraemer 2007; Thorngate, et al. 2002)

Although prolonged stress or ACTH treatment causes adrenal gland hypertrophy, effects on the expression of genes encoding steroidogenic enzymes are less profound (Lehoux, et al. 1998; Ye, et al. 2008). In fact, it may be that induction of intra-adrenal steroid hormone metabolising enzymes help mitigate the effects of excess ACTH. In sheep, for example, there is a marked increase in 20 hydroxylation of corticosteroid intermediates which have no clearly defined biological activity (Butkus, et al. 1985). Similarly, there are reports that adrenal 5 α reductase and sulfotransferase activities may affect the secretion of biologically active hormones (Al-Dujaili, et al. 2011; Luu-The, et al. 2005).

Genomic and somatic mutations of various genes have been identified that explain excess steroid production in cortisol and aldosterone producing adenomas (Calebiro, et al. 2015; Zennaro, et al. 2015). These involve gain/loss of function that affect adrenocortical signalling processes. Although these genes are required, physiological control of steroidogenesis is not necessarily mediated by regulation of their expression. Moreover, there is a need to determine that signalling elements responsible for acute changes in steroid output are the same as those mediating adrenocortical adaptation to chronic stress or prolonged ACTH exposure.

In this study, we have used microarray analysis of mouse adrenal tissue to gain a comprehensive picture of transcriptional control processes affecting cell signalling, cholesterol supply and cell turnover in response to chronic stimulation of corticosterone synthesis in mice infused with an ACTH analogue, Synacthen. We found modest changes in genes encoding steroidogenic enzymes. Our data suggests that enhanced steroidogenic capacity reflects increases in cell size and a shift in the balance between proliferation and apoptosis that increases cell number. Sterol/lipid metabolic pathways are also changed in several ways to allow cholesterol to be channelled towards steroidogenesis.

Materials & Methods

All experiments involving animals were approved by the University of Edinburgh Animal Welfare and Ethical Review Body and were carried out in strict accord with accepted standards of humane animal care under the auspices of the Animal (Scientific Procedures) Act UK 1986. Groups ($n=5/6$) of age-matched male C57BL6 mice (Harlan Olac) weighing approximately 25g were fed a diet containing 0.3% Na (SDS Diets, Witham, Essex, UK) with free access to water in a temperature and light-controlled (12h light/dark cycle; 07.00h lights on) room. Mice were infused sc via miniosmotic infusion pumps (Alzet Cupertino, Model 2002) with either vehicle (0.154M NaCl) or ACTH (Synacthen Ciba-Geigy, UK; 3 μ g/d). At the end of the study mice were killed by carbon dioxide at the nadir of the circadian cycle. Pairs of adrenal glands were collected into a solution of RNeasy (Qiagen Ltd, Crawley, UK). Each adrenal was carefully trimmed free of fat under a dissecting microscope and weighed.

Histology and immunohistochemistry.

Additional groups of mice were infused with ACTH or saline as above to assess effects on adrenal morphology and cell proliferation. Blood was collected by cardiac puncture into lithium heparin tubes for corticosterone measurements by ELISA (Al-Dujaili, et al. 2009). In one experimental cohort, bromodeoxyuridine (1mg/ml) was added to the minipump infusates to monitor cell proliferation. After a two-week infusion, mice were killed by decapitation and tissues collected for fixation in buffered formalin and embedded in paraffin wax. BrdU positive and Ki67 positive nuclei in mid-adrenal sections were located by immunohistochemistry as previously described (Chang, et al. 2013). Cells with Ki67 positive nuclei were counted in the zona glomerulosa/outer zona fasciculata region of the cortex and numbers were normalised to length of capsule perimeter. Haematoxylin and eosin stained

sections were used to estimate cell size in different regions of the adrenal cortex (Chang et al. 2013).

To assess the cellular storage of cholesterol, adrenals were collected without fixation and stored at -80°C before cryosectioning for oil red O staining of lipid droplets. Images were captured with a Niko Coolpix color camera with a Zeiss Axioskop 2 compound microscope and MCID imaging software (Imaging Research Inc, St Catharines, Ontario, Canada)

Apoptosis was analysed using a Roche TUNEL staining kit (Sigma-Aldrich, UK).

Microarray processing

RNA for microarray analysis was prepared from individual adrenal glands (n = 5 and 6 for saline and ACTH-treated mice respectively) using TRIzol® (ThermoFisher Scientific, Loughborough, UK) and then processed through standard Affymetrix protocols, with one round of cDNA amplification (Dunbar, et al. 2010). Processed RNAs from individual adrenal glands were hybridised to Affymetrix Mouse Genome 430 2.0 GeneChip. RNA processing and microarray analyses were carried out by Ark Genomics (Roslin, Edinburgh). Data were analysed as previously described (Dunbar et al. 2010). Microarray data have been archived in the ArrayExpress data repository with the accession number E-MTAB-5704 . Differentially regulated transcripts were analysed with DAVID Bioinformatic Resources (Huang da, et al. 2009) Cluster analysis of genes involved in signalling, sterol and lipid metabolism and cell turnover was carried out with Miru software (Freeman, et al. 2007). Genes of interest that are discussed are listed in supplementary Table 1.

Real time RT-PCR

Based on initial findings from microarrays, selected genes were quantified by pre-optimised RT-PCR assays in RNA from individual adrenals of separate cohorts of saline and ACTH-

treated mice that were killed with CO₂. Total RNA was extracted from tissue samples using the Qiagen RNeasy system and reverse transcribed into cDNA with random primers using the QuantiTect DNase / reverse transcription kit (Qiagen Ltd). cDNA (equivalent to 1ng total RNA) was incubated in triplicate with gene specific primers and fluorescent probes (using pre-designed assays from Applied Biosystems, Warrington, UK) in 1x Roche LightCyclerR480 Probes mastermix. PCR cycling and detection of fluorescent signal was carried out using a Roche LightCyclerR480. A standard curve was constructed for each primer probe set using a serial dilution of cDNA pooled from all samples. Results were corrected for the mean of expression of beta-actin and 18S ribosomal RNA. Neither 18S RNA nor beta actin were affected by ACTH treatment.

Statistics

Data are presented as mean \pm SE. After tests for Gaussian distribution, comparisons were made using either unpaired t-test or ANOVA with Bonferroni post hoc testing; $P \leq 0.05$ were considered statistically significant. Gene expression profiles with Pearson correlation coefficients ≥ 0.9 were analysed for clustering.

Results

Adrenal response to ACTH treatment

Mice infused with ACTH had larger adrenals and higher plasma corticosterone levels (Figure 1A & B). Previous studies of urinary corticosterone excretion in this model indicate that ACTH treatment produces a sustained increase in steroidogenesis (Dunbar et al. 2010). Body weight gain was not affected. After normalisation and correction for multiple testing, microarray analysis indicated that ACTH significantly affected the expression of approximately 9000 gene transcripts ($P < 0.05$; figure 1C); 397 and 531 annotated genes were up- and down-regulated respectively by \geq two-fold ($P < 0.001$). To validate the microarray

results, a parallel analysis was carried out using PCR methods to quantify mRNA of representative genes that were up- and down-regulated in the microarray. The choice of genes reflected a range of responses and their possible involvement in processes controlling signalling and sterol metabolism (*Lpl*, *Srd5a1*, *Scarb1*, *Ren1*, *Mrap*), cell proliferation (*Fgfr11*, *Impdh1*) and apoptosis (*Cidea*, *Casp12*, *Clu*, *Trib3*, *Elmo1*). The patterns of change for the selected genes were broadly similar for microarray and qPCR methods (Figure 1D).

Signalling Genes

Figures 2a and 2b show heat maps of genes implicated in signal transduction that are up- and down-regulated by ACTH treatment ($P < 0.01$). A cluster analysis of signalling genes with Pearson correlation coefficients ≥ 0.9 is shown in supplementary figure 1. In general, the expression patterns of up-regulated genes involved in signalling (figure 2A) were similar to that of the melanocortin receptor accessory protein, *Mrap*. Cross-tabulation of Pearson correlation coefficients for the up-regulated cluster show all values ranged between 0.87 and 0.98. The cluster includes GTPase genes (*Rab2*, *Rab10*, *Rhod*) and genes involved in protein kinase A activity (*Prkar1*, *Prkar2b*) and localisation (*Akap2*). Paradoxically, *Pde8b* was also upregulated. Other genes in the up-regulated cluster were those for factors controlling transcription (*Creb3l2*, *Nr5a1*), cell proliferation and hypertrophy (*Rras2*, *Cdkn1a*, *Rcc2*, *Igflr*, *Shmt1*) and several genes like *Prkcd*, *Srxn1*, *Stx11* and *Inha* with known but ill-defined links to steroidogenesis. The function of others (*Gucalb*, *Fam161a*) have yet to be defined. Analysis of down-regulated signalling genes (figure 2B) suggest secondary events linked to aldosterone/zona glomerulosa functions (*Rgs4*, *Kcnk1*, *Camkk1*, *Shh* and *Prkg2*), neuronal/adrenal medulla tissue (*Insm1*, *Rgs11* and *Kcnq2*) or glucocorticoid activity (*Irs1*, *Atp2b1* and *Scn3b*).

Cholesterol supply

Figure 3A shows the effects of ACTH on oil red O staining suggesting a depletion of cholesterol ester droplets in the zona fasciculata. Microarray data indicated that genes involved in: (i) de novo cholesterol synthesis from acetyl CoA (Figure 3B); (ii) promoting cholesterol uptake from the circulation (Figure 3C); (iii) intracellular cholesterol mobilisation and trafficking and uptake of cholesterol into mitochondria (Figure 3D); (v) disposal of free cholesterol (Figure 3E), were all affected by ACTH treatment. Genes involved in cholesterol synthesis included those encoding HMG CoA reductase (*Hmgcr*) and also squalene epoxidase (*Sqle*) and post-lanosterol enzymes. In keeping with studies demonstrating that cholesterol for steroidogenesis is also derived from the circulation, genes encoding lipoprotein receptors and related proteins that are involved in cholesterol uptake from the circulation were increased. Intracellular cholesterol is trafficked to mitochondria (*Npc1*, *Osbpl6* are upregulated). Genes implicated in the mitochondrial uptake of cholesterol (a rate limiting step in steroidogenesis) were significantly upregulated too although changes in the expression of the key gene, *Star* (1.3 fold), was modest. Other than for immediate steroidogenesis, cholesterol may be stored or disposed. Storage as esters is mediated by transferase enzymes and requires a supply of lipid as well as cholesterol. *Acat1* and *Acat2* (Figure 3E) and a wide range of genes involved in fatty acid synthesis (Supplementary Figure 2) were downregulated. These changes are consistent with reduced oil red O staining.

Genes affecting cholesterol disposal but not directly involved in steroidogenesis were also down-regulated by ACTH. *Cyp27a1*, *Cyp7b1*, *Hsd3b7* genes mediate cholesterol hydroxylation and subsequent isomerase reactions. Overall control of the enzymes and receptors involved in cholesterol synthesis, uptake and metabolism is normally mediated by transcription factors FXR and SREBF1 which in turn are regulated by cholesterol sensing Insig proteins and intracellular sterol levels. *Nr1h4* (*Fxr*), *Serbf1* and *Insig1* genes were all

downregulated. Furthermore, upregulated genes implicated in cholesterol synthesis and uptake cluster together (Supplementary Figure 3). Conversely down-regulated genes associated with cholesterol disposal and lipid biosynthesis form a separate larger cluster.

It is notable, that genes encoding enzymes that metabolise steroids were also affected. *Akr1c18*, which encodes a progesterone 20 α hydroxysteroid dehydrogenase enzyme, was switched on in ACTH-treated adrenals (>80 fold) and expression of *Srd5a1* and 2, which encode 5 α steroid reductase enzymes, were reduced (1.6 and 5.4-fold respectively).

Adrenal Size

Cell Hypertrophy: Part of the ACTH-induced increase in adrenal mass is due to adrenocortical cell hypertrophy. The cross-sectional area of zona fasciculata cells, the main cell type of the cortex, was >2 fold greater but cells of other cortical zones were also increased (figure 4A & B). Medullary cell size was not affected. The expression of representative genes potentially implicated in cell hypertrophy are shown in figure 4C and include factors affecting growth, cell cycle, transcription and protein synthesis.

Cell Hyperplasia: Figures 5A & B show the effects of ACTH on BrdU incorporation and Ki67 positive cells in the adrenal cortex. Labelled cells are located predominantly in the outermost region of the gland. BrdU was incorporated cumulatively over the entire period of infusion whereas Ki67-labelling identified only those cells in S-phase at the time of sacrifice. Immunohistochemistry identified approximately ten times more BrdU-positive than Ki67 - positive cells (Figure 5B). Interestingly, ACTH increased the numbers of BrdU- and Ki67-positive cells to a similar degree indicating that proliferative effects were sustained throughout the period of treatment. Increases in cell proliferation were matched by expression of genes associated with various aspects of cell division (Figure 5C).

Apoptosis: The net effect of ACTH on adrenocortical volume may involve a decrease in cell death as well as increases in cell hyperplasia and hypertrophy (figure 6A & B). Figure 6C and supplementary Figure 4 show the decreased expression of genes with a pattern similar to that of a key apoptotic gene, *Casp12*, and include histocompatibility factors and components of the complement system.

Cluster analysis of genes linked to cell size and turnover (supplementary Figure 5) shows upregulated and downregulated genes. Within the upregulated cluster were overlapping genes associated with hypertrophy and hyperplasia. I

It is notable that genes associated with cell proliferation were more tightly clustered (*Tk1*, *Mki67*, *Cdc2a*). Genes in the downregulated cluster are associated with apoptosis

Discussion

An infusion of ACTH was used to model molecular and morphological changes in the mouse adrenal gland that contribute to long term steroidogenic control. Previously we have reported pathophysiological responses to this treatment, including raised urinary corticosterone to levels which are outwith normal circadian rhythms, thymic involution, hypertension and fluid and electrolyte imbalance, that suggest it represents a model of ACTH-dependent Cushings (Dunbar et al. 2010). The central part of the current study is a microarray analysis of adrenal mRNA expression which established that large numbers of genes were up- and down-regulated indicating that adaptive genomic responses were far wider than might be anticipated from known acute changes in cell signalling and steroidogenesis. Closer analysis of clusters of changes in gene expression indicate that mechanisms are invoked with significant consequences for the continuing supply of steroid hormone substrate (cholesterol), for adrenocortical cell kinetics, for adrenocortical cell hypertrophy and for processes that might compensate excess glucocorticoid hormone. Genes encoding signalling factors that are responsible for these functional changes were also noted.

The obligatory role for cholesterol in steroidogenesis and the factors and genetic diseases involving cholesterol metabolism which affect steroidogenesis are well recognised and have been reviewed extensively (Kraemer 2007; Miller and Bose 2011). Sustained high corticosterone output requires continuing cholesterol supply rather than mobilisation of extant stores. This need is met by *de novo* synthesis and by uptake from the circulation. Genes encoding enzymes involved in cholesterol synthesis, including *Hmgcr*, *Sqle*, *Scd5*, *Hsd17b7*, *Dhcr7*, are up-regulated. It should be noted however that enzymes encoded by these genes and other enzymes in cholesterol biosynthesis are also regulated non-genomically and are subject to post-translational modification with activities controlled by kinases and various

sterols (Sharpe and Brown 2013). It is perhaps significant that *Insig1* that encodes a cholesterol sensor, is down-regulated more than twofold by ACTH. *Insig1* reduces transcriptional activity and promotes degradation of key enzymes involved in cholesterol biosynthesis like HMG-CoA reductase (Dong, et al. 2012).

Adrenal cholesterol is also provided by circulating lipoproteins: predominantly LDL in humans via LDL receptors (*Ldlr*) and HDL in rodents via scavenger receptors (*Scarb1*) (Gwynne and Strauss 1982; Kraemer 2007; Rigotti, et al. 1996). ACTH increased expression of both *Ldlr* and *Scarb1* as well as *Ldlrap1* which facilitates LDL uptake. *ApoE*, which is also known to affect LDL receptor activity and is highly expressed specifically in the adrenal cortex, was not affected. This contrasts with previous reports on apolipoprotein E protein and mRNA levels (Cheng, et al. 1998; Prack, et al. 1991). However, the gene *Lrp8* which encodes a receptor for ApoE was upregulated as was *Vldlr*; both genes have been associated with the reelin signalling system which controls neural development and plasticity (Herz and Chen 2006) suggesting an alternative adrenal function. Similarly, *Hdlbp*, although increased by ACTH treatment and with an affinity for HDL (Fidge 1999), is also known as vigilin, an RNA binding protein with wider functions that might be independent of steroidogenesis (Mobin, et al. 2016).

Cholesterol from circulating lipoproteins is taken up in the form of esters requiring hydrolysis to render free cholesterol for steroidogenesis. Two genes encoding adrenal cholesterol esterases have been identified: hormone sensitive lipase (*Lipe*) (Kraemer 2007) and more recently neutral cholesterol ester hydrolase (*Nceh1*) (Ohta, et al. 2011). Control of hormone sensitive lipase activity may be post-translational (Yeaman 2004); *Lipe* expression is not significantly affected in the present study (-1.67, P = 0.1). In contrast, *Nceh1* is modestly

increased by ACTH treatment (1.82, $P < 0.0001$). It is notable that genes encoding factors required for intracellular cholesterol trafficking (*Npc1*, *Stx11*) (Li, et al. 2016; Lin, et al. 2016) were also up-regulated.

Under normal conditions, excess cholesterol is stored as esters in lipid droplets. As evidenced previously (Cheng et al. 1998; Thorngate et al. 2002) and here by oil red O staining, the zona fasciculata cells of ACTH-treated mice are depleted of lipid. A pattern of reduced expression of genes involved in cholesterol and lipid metabolism is consistent with this depletion. *Plin4* (perilipin 4, a component of the droplet coat) (Kraemer, et al. 2013) is down-regulated as are *Acat1* and 2, the transferases that esterify cholesterol. However, these transferases are also known to be allosterically activated by sterols (Rogers, et al. 2015). In addition, many genes implicated in the biosynthesis of the lipids which are co-substrates in cholesterol ester synthesis are down-regulated, implying that triglycerides are normally produced locally to maintain cholesterol ester reserves (Supplementary Figure 2).

A further way of optimising cholesterol supply is to limit non-steroidogenic routes of metabolism. Excretion of cholesterol is via hepatic bile acid synthesis and although the adrenal does not produce significant amounts of bile, genes encoding three genes in bile synthesis are down-regulated as is *Fxr*, a key transcription factor regulating the bile pathway (Russell 2009). *Stard10* which binds phosphatidyl choline and is involved in bile acid metabolism, is also down-regulated (Alpy and Tomasetto 2014). The work of Schroeder et al (Schroeder, et al. 2010) has implicated sterol carrier protein-2 (*Scp2*) and caveolin-1 (*Cav1*) as factors in liver controlling the intracellular distribution, efflux and esterification of cholesterol. It is significant therefore that ACTH downregulated both *Scp2* (-1.62 fold, $P < 0.001$) and *Cav1* (-2.37 fold, $P < 0.0001$) expression.

The first step in steroidogenesis is the mitochondrial side chain cleavage of cholesterol to produce pregnenolone. Cleavage activity is determined by cholesterol uptake across the mitochondrial membrane. Papadopoulos and colleagues have suggested that this involves a complex of five proteins termed a transduceosome (Liu, et al. 2006). Although this hypothesis is controversial (Selvaraj, et al. 2016), of the genes encoding these five proteins, *Vdac1* and *Prkar1a* are upregulated two fold ($P < 0.001$), *Star* and *Acbd3* are slightly increased (1.2 fold, $P < 0.01$) and *Tspo* (peripheral benzodiazepine receptor) expression is unaffected.

The relative change in genes encoding steroidogenic enzymes is modest (< 1.3 fold) reaffirming studies that show cholesterol supply is critical in maintaining high corticosteroid output when enzyme expression is non-limiting. However, it is notable that ACTH has profound effects on other genes affecting steroid metabolism. *Akr1c18*, which encodes a 20α hydroxysteroid dehydrogenase enzyme, was switched on in ACTH-treated adrenals (> 80 fold). This is consistent with observations in sheep where circulating levels of dihydroxyprogesterone were increased in ACTH-induced hypertension (Butkus et al. 1985). Previous studies in mouse indicate that adrenal *Akr1c18* expression is normally high in prepubertal and low in adult males (Hershkovitz, et al. 2007). In contrast expression of *Sd5a2* that encode a 5α steroid reductase, which is normally high in the male mouse adrenal gland (Luu-The et al. 2005), was decreased more than 5-fold by ACTH. In general, the activity of these enzymes controls the availability of intermediates in the corticosteroid pathway thereby affecting throughput to biologically active hormonal end products.

A major contribution to steroidogenic capacity is the marked threefold increase in adrenal mass. The morphology of adrenals from ACTH-treated mice indicate this is due to expansion

of the cortex rather than the medulla and is a function of both the size and numbers of parenchymal cells. Histology shows that cell hypertrophy is predominantly a feature of the zona fasciculata. The twofold increase in cross-sectional area with ACTH treatment is perhaps an underestimate since volume expansion is offset by depletion of lipid droplets. Increased expression of *Cdkn1a*, a marker of cell cycle arrest, suggested cell cycle progression beyond the G1 stage is inhibited in some cells. The close correlation of *Cdkn1a* expression with other genes (correlation coefficient >0.9 , $P < 0.0001$) reflects changes in growth and de novo protein. *Cdkn1c*, a gene known to affect adrenal cell turnover in embryonic life (Nagahama, et al. 2001), was also increased (4.12 fold) but with a pattern of change that did not strongly correlate with that of *Cdkn1a* ($r = 0.62$).

BrdU and Ki67 immunostaining demonstrated ACTH caused a sustained stimulation of cell division which appeared to be initiated in a subcapsular region of the gland with displacement of newly divided cells inwards (Chang et al. 2013). The majority of hypertrophied cells throughout the cortex were not immunostained suggesting that increased cell size is independent of mitosis. In line with Ki67 staining, expression of *Mki67* was also increased as were a number of genes linked to cell proliferation and the cell cycle. *Tk1*, encoding thymidine kinase, another established marker of proliferation, was increased 2.25 fold ($P < 0.0001$). As with genes controlling cell hypertrophy, expression of genes associated with proliferation were closely clustered.

Cell number is determined by cell death (apoptosis) as well as division. The term apoptosis was first used in the seminal work of Kerr et al (Kerr, et al. 1972) to describe programmed cell death in several experimental models including the effects of ACTH withdrawal on the rat adrenal cortex. Not surprisingly, ACTH infusion in the present experiment had anti-

apoptotic effects which were linked to decreased expression of genes like *Casp12*. These genes include histocompatibility factors, which previous studies have shown are expressed in inner regions of the cortex with a role in apoptosis (Wolkersdorfer, et al. 1996). Similarly, various components of complement activation that have been shown to play a role in the clearance of apoptotic cells (albeit in non-adrenal cells (Ogden and Elkon 2006)) were decreased. It should be stressed, however, that the level of apoptosis in a normal gland is low so that an ACTH-induced reduction in apoptosis may be difficult to detect, particularly if cell clearance is efficient (Carsia, et al. 1996). Nevertheless, TUNEL staining in the present study showed a fivefold decrease with ACTH treatment. This is perhaps an overestimate given that the number of apoptotic cells is expressed relative to cross sectional area of the cortex and the cross-sectional area of cells from ACTH-treated adrenals is up to two times greater than controls.

The diversity of cellular effects underlying the response to chronic ACTH treatment is reflected in the range of genes involved in signalling. ACTH receptor regulation of adrenal activity is mediated through the cyclic AMP cascade (Gallo-Payet 2016) but few of the genes involved are affected. Adenylate cyclase isoforms are generally down-regulated and *Pde8b*, which is negatively associated with steroidogenic activity (Horvath, et al. 2008), was up-regulated by ACTH treatment. Genes involved in protein kinase A activity which are mutated in some patients with ACTH-independent Cushings (Calebiro et al. 2015) were up-regulated as was the protein kinase A anchor protein *Akap2*. The upregulated cluster was enriched with genes implicated in processes downstream of the cyclic AMP cascade including genes for transcription factors (*Nr5a1*, *Creb3l2*), for cell proliferation and hypertrophy (*Rras2*, *Cdkn1a*, *Rrcc2*, *Igflr*, *Shmt1*) and for snare proteins (*Stx11*). Genes like *Prkcd*, *Srxn1* and *Inha* are known to be involved in steroidogenic control (Brizuela, et al. 2006; Hofland and de Jong

2012; Kil, et al. 2012) whereas the function of others (*Gucalb*, *Fam161a*) have yet to be defined.

Analysis of down-regulated signalling genes suggest secondary events. For example, *Rgs4*, *Kcnk1*, *Camkk1*, *Shh* and *Prkg2* have been linked to aldosterone/zona glomerulosa functions (Azizan, et al. 2012; Beuschlein, et al. 2013; Nanba, et al. 2015; Romero, et al. 2007; Spiessberger, et al. 2009; Walczak, et al. 2014), *Insm1*, *Rgs11* and *Kcnq2* are associated with neuronal/ adrenal medulla tissue (Anderson, et al. 2009; Etxeberria, et al. 2008; Wildner, et al. 2008) and expression of *Irs1*, *Atp2b1* and *Scn3b* are controlled by glucocorticoids (Bhargava, et al. 2002; Fahmi, et al. 2004; Giorgino, et al. 1993; Turnbow, et al. 1994).

In summary, a large number of adrenal genes are differentially affected by chronic ACTH treatment. Upregulated genes include those implicated in cell division and cell hypertrophy as well as those promoting cholesterol supply from de novo synthesis and uptake from the circulation. Downregulated genes are those involved in apoptosis, intracellular storage of cholesterol esters and non-steroidogenic metabolism of cholesterol. Preliminary observations indicate that genes controlling each of these different processes are co-ordinately regulated. Theoretically, this information could be used to target genes to manipulate glucocorticoid output or ACTH responsiveness. For example, the adverse effects of glucocorticoid hormones could be ameliorated in patients with Cushing's disease due to excess pituitary ACTH or Cushing's Syndrome caused by ectopic ACTH production. It is important, however, to reiterate that many genes that are transcriptionally regulated by ACTH encode factors which are subject to non-genomic control. Finally, excessively high glucocorticoid hormone levels may, secondarily, affect gene expression in the zona glomerulosa and the adrenal medulla with consequences for aldosterone and catecholamine synthesis.

Declaration of Interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research.

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Contributions

Conception: CJK, JJM, LJM, DRD; Execution: RIM, DRD, LJM, XZ, NW, CC, CJK;

Interpretation: DRD, MAB, RIM, JJM, LJM, CJK; Manuscript preparation: RIM, LJM, MAB, JJM, DRD, CJK.

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Figure Legends

Figure 1: ACTH infusion causes adrenal hypertrophy (A), increased corticosterone levels (B) and altered expression of gene transcripts (C & D). Values shown in A and B are mean values \pm SEM, n = 6. Microarray values (grey bars) are compared with RT-PCR values (black bars) for a range of up and down regulated genes (D).

Figure 2. Heat maps showing expression genes involved in cell signalling that are up-regulated (A) and down-regulated (B) by ACTH. Each square represents gene expression of a single sample. Shades of blue and red indicate levels of expression below and above normalised values for individual genes.

Figure 3: ACTH infusion causes depletion of lipid droplets in adrenal cortex (A) and up-regulation of gene transcripts associated with cholesterol biosynthesis (B), the cellular uptake of cholesterol (C) and the intracellular distribution of cholesterol (E). Down-regulated transcripts associated with non-steroidogenic routes of cholesterol metabolism are shown (D). Values are means \pm SEM of n =5 (control) and 6 (ACTH) adrenal glands.

Figure 4: ACTH infusion caused adrenocortical cell hypertrophy (A & B) and increased expression of transcripts associated with cell growth (C). (A) shows representative H&E stained sections with bar indicating magnification (100 μ m). (B) shows cross sectional area of cells in zona glomerulosa (ZG), outer zona fasciculata (OZF), inner zona fasciculata (IZF) and medulla (MED) regions of the gland. Values are means \pm SEM of n =6 (control) and 6 (ACTH) adrenal glands. (C) shows increased expression of genes associated with cell size.

Figure 5: ACTH infusion increased adrenocortical cell proliferation (A & B) and the expression of gene transcripts associated with cell division (C). Sections of adrenal gland

from control and ACTH were dual immunostained (A) for Ki67 (brown nuclei) and bromodeoxyuridine (purple nuclei) and analysed for numbers of positive nuclei (B). Values are means \pm SEM of $n = 6$ (control) and 6 (ACTH) adrenal glands; * - $P < 0.01$, ** - $P < 0.001$). ACTH-induced fold changes in gene transcripts associated with cell proliferation are shown in (C).

Figure 6: ACTH treatment reduced TUNEL staining in the adrenal cortex (A). Numbers of apoptotic cells corrected for cross-sectional area of cortex (B) were significantly reduced ($P < 0.001$). Values shown are means \pm SEM of $n = 4$ control and ACTH-treated adrenals. ACTH decreased expression (fold change) associated with apoptosis are shown in (C) (see also supplementary figure 2).

Supplementary Information

Supplementary Figure 1: Cluster analysis of adrenal signalling genes that are controlled by ACTH. Lines represent Pearson correlation coefficients for associations between genes that are ≥ 0.9 . The cluster on the left comprises up-regulated genes; the cluster on the right are down-regulated genes.

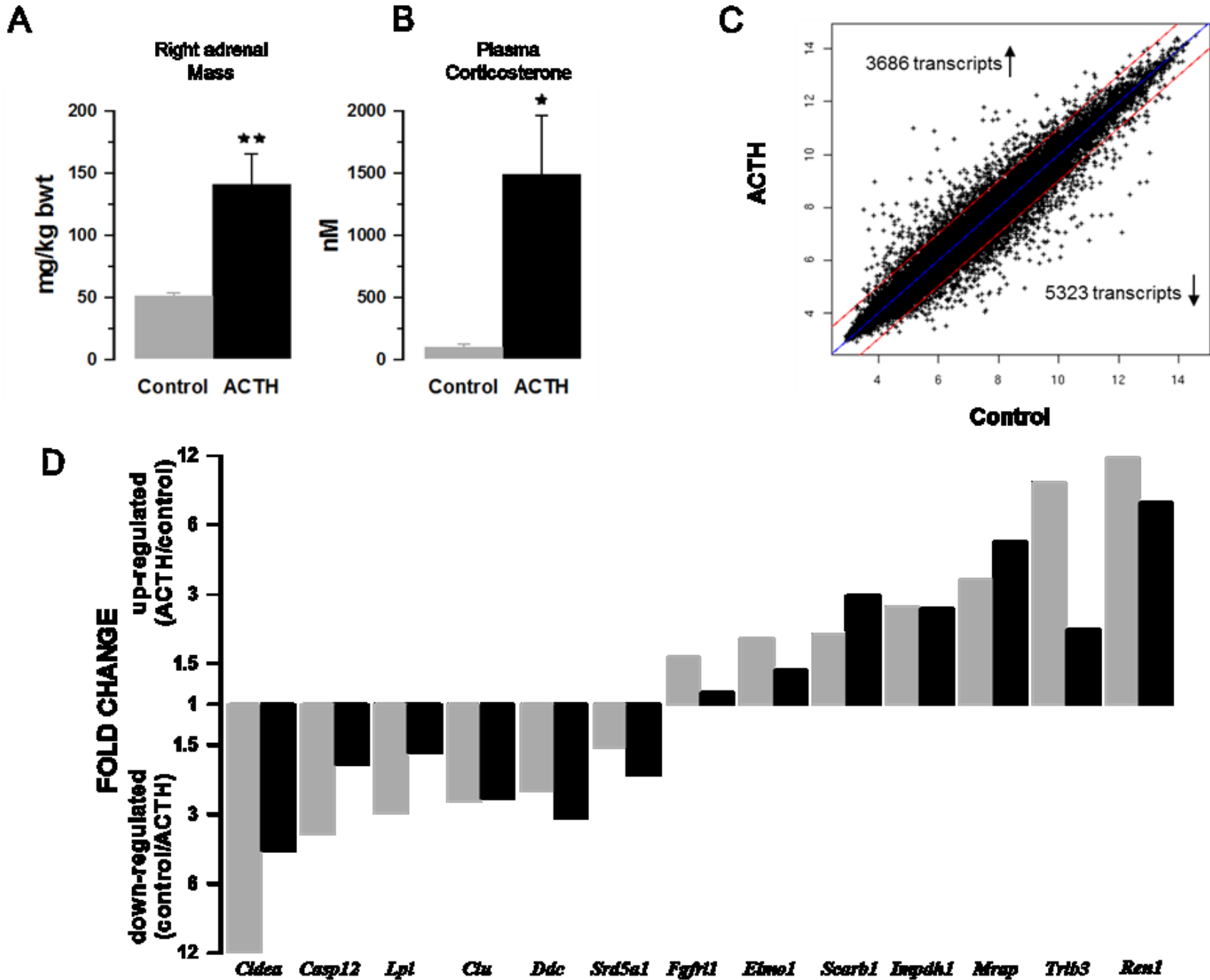
Supplementary Figure 2: Heat map showing genes associated with the control of lipid biosynthesis which are down-regulated by ACTH treatment. Each square represents gene expression of a single sample. Shades of blue and red indicate levels of expression below and above normalised values for individual genes.

Supplementary figure 3: Cluster analysis of adrenal genes controlled by ACTH that are implicated in sterol/lipid metabolism. Lines represent Pearson correlation coefficients between genes that are ≥ 0.9 . The cluster on the upper left comprises up-regulated genes including those involved in cholesterol biosynthesis (eg *Sqle*, *Hsd17b7*), uptake from the circulation (eg *Scarb1*) and intracellular trafficking (eg *Npc1*, *Vdac1*). The cluster on the lower right are down-regulated genes involved in cholesterol storage (eg *Acat1*, *Plin4*) and disposal (*Cyp27a1*) and lipid biosynthesis (eg *Adipoq*, *Ppara*, *Fasn*).

Supplementary Figure 4: Heat map showing the effects of ACTH treatment on down-regulated genes associated with apoptosis. Each square represents gene expression of a single sample. Shades of blue and red indicate levels of expression below and above normalised values for individual genes.

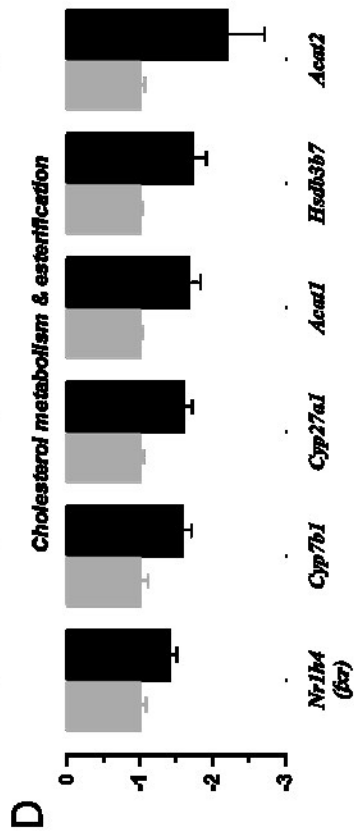
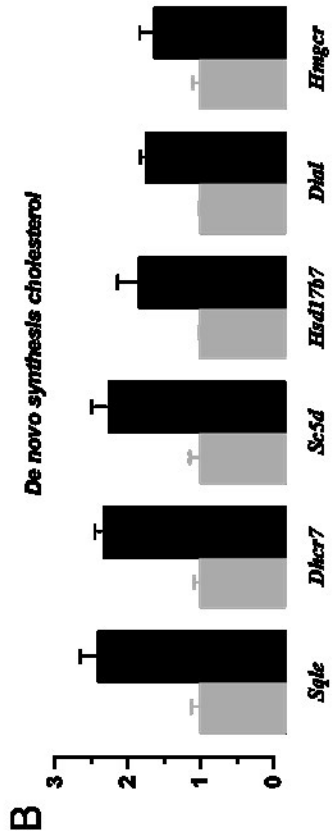
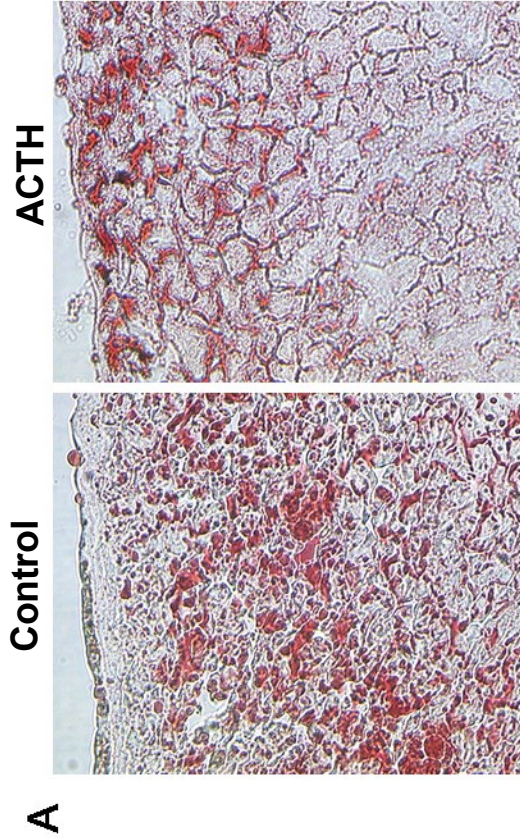
Supplementary Figure 5: Cluster analysis of adrenal genes modulated by ACTH treatment that are associated with cell hyperplasia, hypertrophy and apoptosis. Lines represent Pearson correlation coefficients between genes that are ≥ 0.9 . The cluster on the left comprises up-regulated genes that are associated with cell hyperplasia and hypertrophy: note the genes in grey include *Mki67*, *Tk1* and *Cdc2a* that are involved in cell proliferation. The genes in yellow on the right are down-regulated genes including *Casp12* and various histocompatibility and complement system genes that are associated with apoptosis.

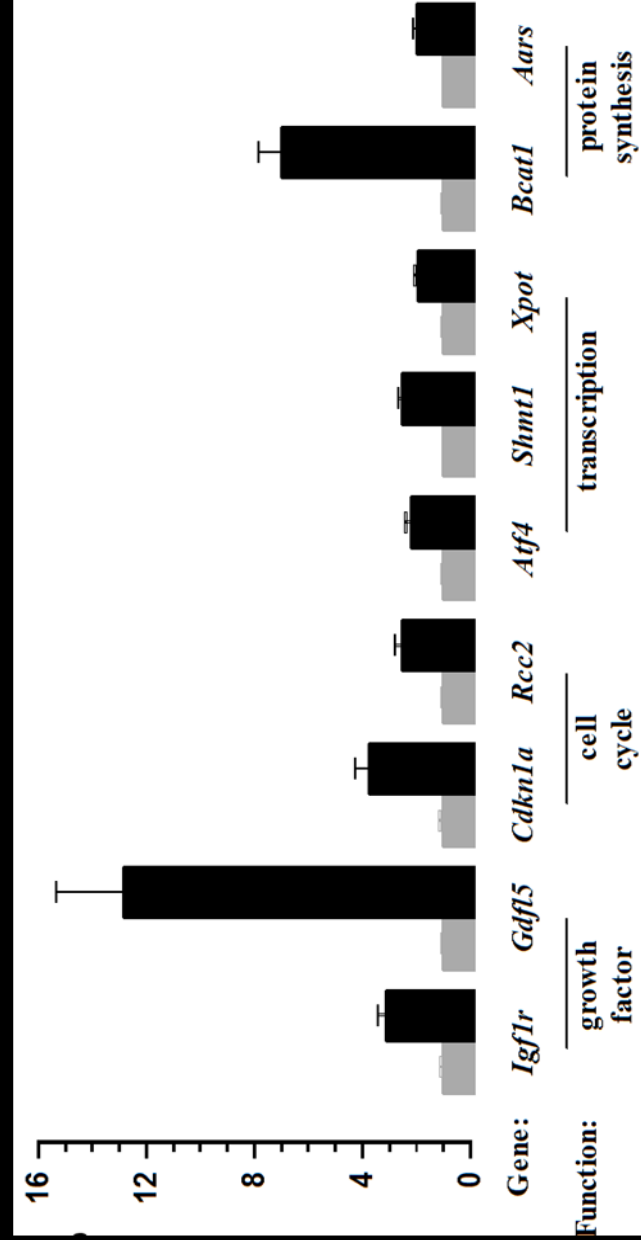
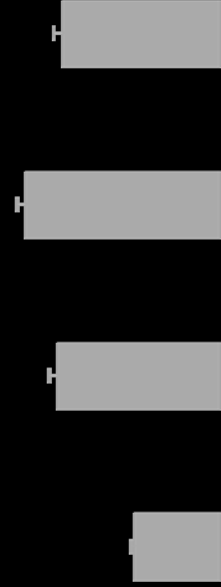
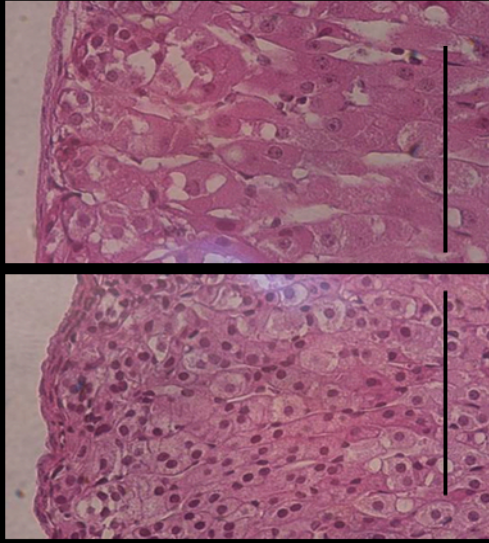
Supplementary Table1: Genes of Interest

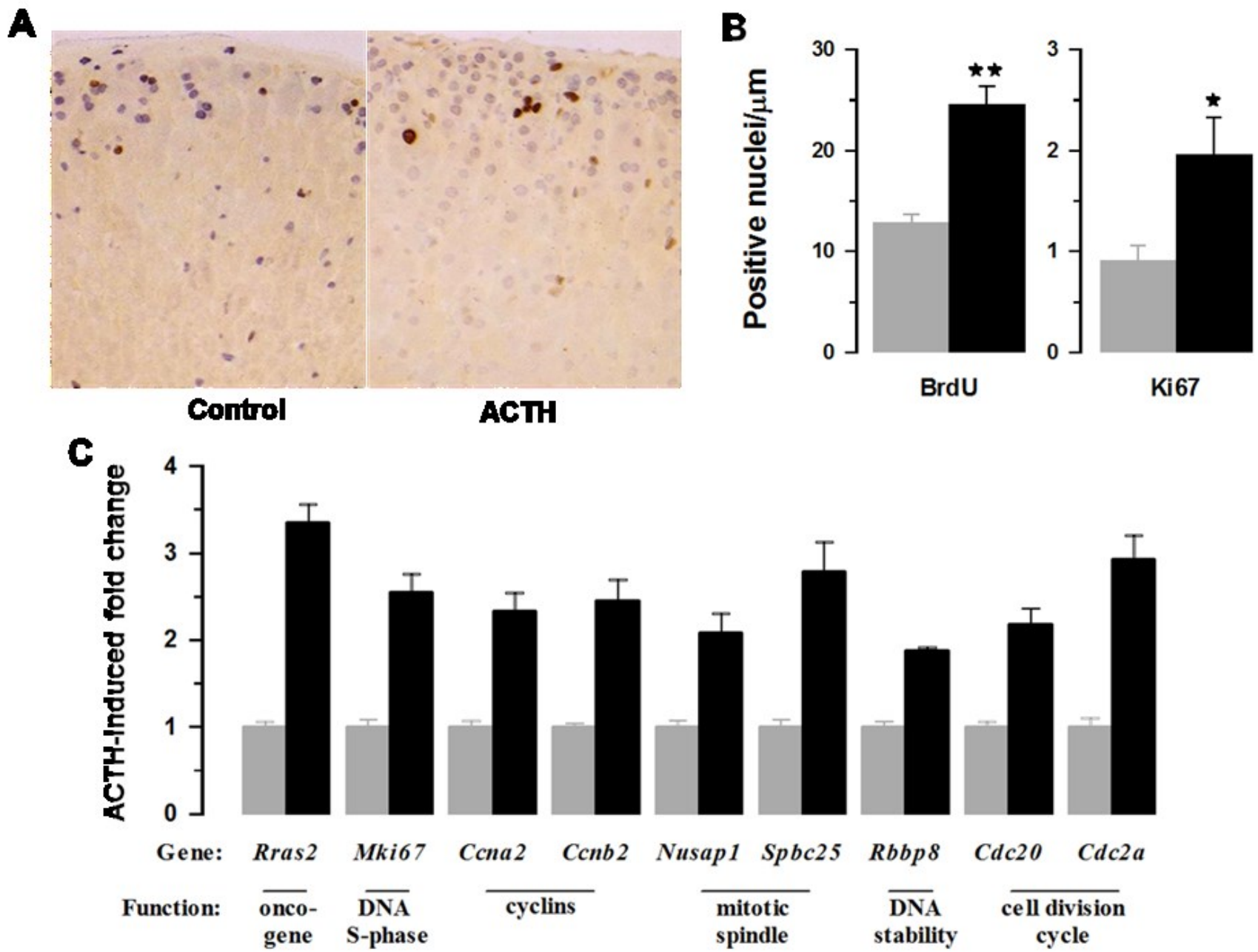


Gene	Control	ACTH	Fold Upregulated	P-value
<i>mrp</i>			3.43 ± 0.25	< 0.0001
<i>prkcd</i>			2.54 ± 0.07	< 0.0001
<i>impdh1</i>			2.66 ± 0.15	< 0.0001
<i>rab10</i>			2.04 ± 0.04	< 0.0001
<i>sdcd3</i>			2.41 ± 0.17	< 0.0002
<i>creb3l2</i>			2.09 ± 0.17	< 0.0003
<i>prkar2b</i>			2.42 ± 0.18	< 0.0004
<i>rab2b</i>			3.08 ± 0.37	< 0.001
<i>rhod</i>			2.68 ± 0.18	< 0.0001
<i>rcc2</i>			2.53 ± 0.25	< 0.0005
<i>inha</i>			5.61 ± 0.74	< 0.0001
<i>akap2</i>			2.21 ± 0.11	< 0.0001
<i>mapk13</i>			2.73 ± 0.34	< 0.002
<i>sdcd1</i>			6.57 ± 0.66	< 0.0001
<i>guca1b</i>			2.22 ± 0.19	< 0.0004
<i>nr5a1</i>			2.22 ± 0.18	< 0.0004
<i>cdkn1a</i>			3.75 ± 0.51	< 0.002
<i>igf1r</i>			3.10 ± 0.32	< 0.003
<i>shmt1</i>			2.52 ± 0.15	< 0.0001
<i>rras2</i>			3.36 ± 0.20	< 0.0001
<i>stx11</i>			4.06 ± 0.46	< 0.0003
<i>Srxn1</i>			11.61 ± 1.40	< 0.0001
<i>fam161a</i>			5.89 ± 0.31	< 0.0001
<i>pde8b</i>			2.86 ± 0.34	< 0.001
<i>prkar1a</i>			1.61 ± 0.05	< 0.0001

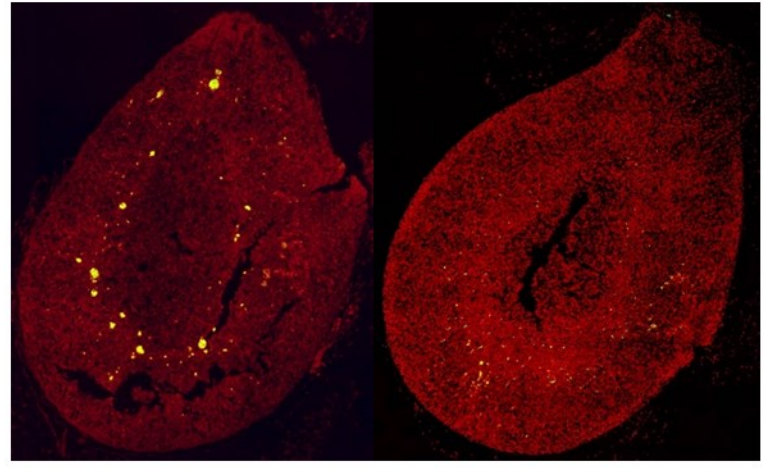
Gene	Control	ACTH	Fold Downregulated	P-value
<i>rgs11</i>			3.99 ± 0.15	< 0.0001
<i>kcnk1</i>			3.70 ± 0.28	< 0.0001
<i>kctd14</i>			3.14 ± 0.16	< 0.0001
<i>camkk1</i>			2.11 ± 0.15	< 0.0003
<i>osbpl3</i>			4.54 ± 0.42	< 0.0001
<i>jun</i>			2.79 ± 0.48	< 0.02
<i>atp2b3</i>			2.10 ± 0.11	< 0.0001
<i>scn3b</i>			2.44 ± 0.22	< 0.0004
<i>shh</i>			3.34 ± 0.32	< 0.0002
<i>prkg2</i>			4.59 ± 0.56	< 0.0003
<i>rgs4</i>			3.41 ± 0.25	< 0.0001
<i>slc12a2</i>			3.39 ± 0.34	< 0.0003
<i>stx17</i>			2.00 ± 0.07	< 0.0001
<i>adcy7</i>			2.43 ± 0.32	< 0.005
<i>kcnq2</i>			2.23 ± 0.19	< 0.0003
<i>ccbe1</i>			3.54 ± 0.48	< 0.005
<i>insm1</i>			3.72 ± 0.52	< 0.005
<i>irs1</i>			3.14 ± 0.11	< 0.0001
<i>igfbp3</i>			3.70 ± 0.35	< 0.0002
<i>atp8a1</i>			2.54 ± 0.40	< 0.0001
<i>atp2b1</i>			2.15 ± 0.34	< 0.0001







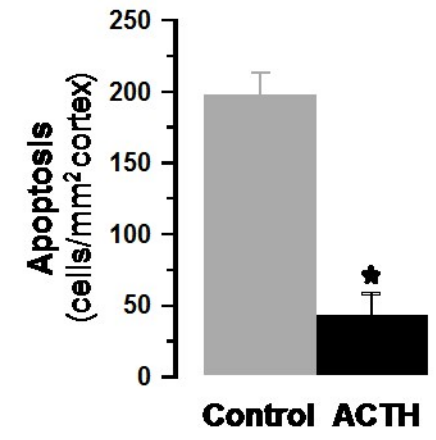
A



Control

ACTH

B



C

